

**MANIPULATION OF PROBIOTICS FERMENTATION OF  
MILK BY *CINNAMON ZEYLANICUM*, *GLYCYRRHIZA  
GLABRA* OR *ALLIUM SATIVUM* AND THEIR EFFECTS  
ON INHIBITION OF *HELICOBACTER PYLORI* GROWTH  
*IN VITRO***

**SARA BEHRAD**

**FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

**2011**

**MANIPULATION OF PROBIOTICS FERMENTATION OF  
MILK BY *CINNAMON ZEYLANICUM*, *GLYCYRRHIZA  
GLABRA* OR *ALLIUM SATIVUM* AND THEIR EFFECTS  
ON INHIBITION OF *HELICOBACTER PYLORI* GROWTH  
*IN VITRO***

**SARA BEHRAD**

**THESIS SUBMITTED IN FULFILMENT  
OF THE REQUIREMENTS FOR  
THE DEGREE OF MASTER OF SCIENCE**

**FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

**2011**

# ACKNOWLEDGMENT

I would like to thank all people who have helped, inspired and supported me in any respect during the completion of this project.

I am heartily thankful to my supervisor, *Associate Professor Dr. Ahmad Salihin Baba*, whose encouragement, guidance and support from the initial to the final phases enabled me to grow and develop. I appreciate him for giving me a challenging and motivating research project which include the disciplines of Biochemistry, Medical Microbiology and Food Biotechnology.

I would like to express my sincere gratitude to *Prof. Dato' Dr. KL Goh*, the head of Gastroenterology unit, Faculty of Medicine, for his valuable advice and friendly help.

I warmly thank *Prof. Mohd Yasim Mohd Yusof* from Microbiology Department, University of Malaya, who has guided me and allowed me to use the facilities in Microbiology Lab. Special thanks also goes to *Ms. Chan Chui Lin* for her assistance in the isolation of *H. pylori* isolates.

I take this opportunity to express my profound gratitude to my beloved families; especially my mother, grandfather and my aunt for their unflagging love and endless support through my life. Words alone can't express my sense of gratitude; simply "God blessed me to have you around and thank you".

Many thanks to my dearest husband, whose patience, accompany, love, and support enabled me to complete this project.

Thanks to all my friends and lab mates for helpful discussions and support.

Last but not least, thanks to God for guiding my life through all tests in the past three years. You have blessed me and made my life more bountiful. May your name be honored and glorified.

Thank you  
Sara Behrad  
November 2010

# ABSTRACT

Dairy products containing probiotics (e.g. *Lactobacillus* ssp. and *Bifidobacterium*) and certain herbs have inhibitory effects on the growth of *Helicobacter pylori*. The objectives of the present study were to determine the effects of herbs traditionally used for the treatment of gastric ulcer on yogurt fermentation characteristics, probiotic bacteria and the growth of *H. pylori* *in vitro*. Cinnamon zeylanicum (cinnamon), *Glycyrrhiza glabra* (licorice) or *Allium sativum* (garlic) was individually mixed with milk and the mixtures were fermented by probiotic bacteria to form herbal-yogurts. Changes in pH, titratable acidity, anti oxidant activity and the viable cell count of *Lactobacillus* ssp. and *Streptococcus thermophilus* were evaluated during refrigerated storage. The *in vitro* inhibition of *H. pylori* growth was determined using agar diffusion and minimum inhibitory concentration (MIC) methods. There were no significant differences in pH and TA between herbal-yogurts and plain-yogurt during fermentation and storage. Refrigeration up to 28 days increased ( $p>0.05$ ) viable *Lactobacillus* ssp. counts to  $15.8 \times 10^6$  cfu/ml in the plain-yogurt but the presence of cinnamon, licorice or garlic tend to inhibit the increase ( $p>0.05$ ) in herbal yogurts. Garlic-yogurt showed the least favored ( $p>0.05$ ) for all characteristics tested for organoleptic properties. Water extract of cinnamon-yogurt and licorice-yogurt on day 7 of refrigerated storage showed the highest inhibitory effect against *H. pylori* strains UM-1, UM-2 and UM-3. An MIC of 3ml was effective for all *H. pylori* isolates by cinnamon and licorice yogurt water extracts, but only for *H. pylori* isolate UM-3 by garlic yogurt extract. Licorice-yogurt water extract had MIC at 1ml for isolate UM-1 and UM-2, whereas cinnamon-yogurt water extract had MIC at 2ml. Garlic-yogurt water extract had weak inhibition on *H. pylori*. The present *in vitro* findings indicate that yogurt and herbs under study can decrease the growth of *H. pylori*. These herbs could be used as food additives for the production of novel dairy products because of their unique functional attributes and potential mitigation on *H. pylori* growth.

*Key words: Probiotics – Helicobacter pylori – Medicinal Herbs – Herbal-yogurt – Lactobacillus* ssp.

# ABSTRAK

Produk-produk tenusu yang mengandungi probiotik (misalnya ssp. *Lactobacillus* dan *Bifidobacterium*) dan herba tertentu mempunyai kesan menghalang pada pertumbuhan *Helicobacter pylori*. Penelitian ini adalah untuk mengaji kesan dari herba yang digunakan secara tradisi untuk rawatan ulser perut ke atas ciri-ciri fermentasi yogurt, bakteria probiotik dan pertumbuhan *H. pylori in vitro*. *Cinnamon zeylanicum* (kayu manis), *Glycyrrhiza glabra* (licorice) atau *Allium sativum* (bawang putih) dicampur berasingan dengan susu dan campuran difermentasikan oleh bakteria probiotik untuk membentuk yogurt herba. Perubahan pH, jumlah asid tertitrasi, aktiviti anti oksidan dan jumlah sel hidup *Lactobacillus* ssp dan *Streptococcus thermophilus* dinilai selama simpanan berpendingin. Perencatan pertumbuhan *in vitro H. pylori* ditentukan dengan menggunakan kaedah difusi agar dan kepekatan minimum perencatan (MIC). Tidak ada perbezaan yang signifikan pada pH dan TTA antara yogurt herba dan yogurt kawalan selama fermentasi dan simpanan berpendingin. Pendinginan sehingga 28 hari meningkat ( $p > 0.05$ ) *Lactobacillus* ssp ke jumlah  $15.8 \times 10^6$  cfu/ml untuk yogurt kawalan namun kewujudan kayu manis, licorice atau bawang putih cenderung menghalang peningkatan ( $p > 0.05$ ) pada yogurt herba. Yogurt bawang putih adalah paling tidak disukai ( $p > 0.05$ ) untuk semua ciri-ciri diuji untuk sifat organoleptik. Ekstrak air yogurt kayu manis dan yogurt licorice pada 7 hari simpanan berpendingin menunjukkan kesan penghambatan yang tinggi terhadap strain *H. pylori* UM-1, UM-2 dan UM-3. Ekstrak yogurt kayu manis dan yogurt licorice pada MIC 3ml adalah berkesan merencat semua isolat *H. pylori* tetapi hanya pada MIC 3ml bagi ekstrak yogurt bawang putih. Ekstrak air yogurt licorice pada kepekatan 1ml berupaya merencat UM-1 dan UM-2, sedangkan air ekstrak yogurt kayu manis mempunyai MIC 2ml. Ekstrak air yogurt bawang putih menghalang pertumbuhan *H. pylori*. Yogurt dan herba-herba yang diteliti secara *in vitro* dapat merencat pertumbuhan *H. pylori*. Tumbuh-tumbuhan ini boleh digunakan sebagai makanan tambahan untuk pengeluaran produk tenusu baru kerana sifat unik berfungsi dan potensinya mengekang pertumbuhan *H. pylori*.

# Table of Contents

	Page
AKNOWLEDGMENT .....	III
ABSTRACT.....	IV
ABSTRAK .....	V
LIST OF ABBREVIATIONS .....	XI
CHAPTER 1: INTRODUCTION .....	1
CHAPTER 2: LITERATURE REVIEW .....	4
2.1 YOGURT .....	4
2.1.1 HEALTH BENEFITS OF YOGURT.....	4
2.2 THE HUMAN GASTROINTESTINAL TRACT AND ITS MICROBIOTA .....	6
2.3 PROBIOTICS .....	7
2.3.1 INTRODUCTION .....	7
2.3.2 THERAPEUTIC VALUE OF PROBIOTICS .....	8
2.3.2.1 <i>Improvement of lactose metabolism</i> .....	9
2.3.2.2 <i>Reduction in serum cholesterol level</i> .....	9
2.3.2.3 <i>Immune system stimulation</i> .....	9
2.3.2.4 <i>Anticarcinogenic and antimutagenic activity</i> .....	10
2.3.2.5 <i>Controlling gastrointestinal infections</i> .....	10
2.3.2.6 <i>Suppression of Helicobacter pylori infection</i> .....	11
2.3.3 IMPORTANT CRITERIA FOR PROBIOTIC LACTIC ACID BACTERIA .....	11
2.4. LACTIC ACID BACTERIA (LAB).....	12
2.4.1 ANTIBACTERIAL COMPONENTS FROM LACTIC ACID BACTERIA .....	13
2.4.1.1 <i>Organic acids</i> .....	14
2.4.1.2 <i>Hydrogen peroxide</i> .....	14
2.4.1.3 <i>Carbon dioxide</i> .....	14
2.4.1.4 <i>Bacteriocins</i> .....	14
2.4.1.5 <i>Adhesion inhibitors</i> .....	15
2.5 HERBS.....	15
2.5.1 INTRODUCTION .....	15
2.5.2 CINNAMON ZEYLANICUM .....	16
2.5.2.1 <i>Medicinal value of cinnamon</i> .....	17
2.5.3 GLYCYRRHIZA GLABRA .....	18
2.5.3.1 <i>Medicinal value of licorice</i> .....	19
2.5.4 ALLIUM SATIVUM .....	20

2.5.4.1 Medicinal value of garlic.....	21
2.6 <i>HELICOBACTER PYLORI</i> .....	21
2.6.1 DISEASE AND PREVALENCE.....	21
2.6.2 CHARACTERISTICS AND GROWTH REQUIREMENTS OF THE GENUS <i>HELICOBACTER PYLORI</i> .....	23
2.6.3 MODE OF TRANSMISSION .....	24
2.6.3 MECHANISM OF ADHESION TO THE STOMACH CELLS.....	25
2.6.4 THERAPY .....	27
CHAPTER 3: MATERIALS AND METHODS .....	29
3.1 MATERIALS .....	29
3.1.1 MEDICINAL HERBS.....	29
3.1.2 <i>Yogurt bacteria and probiotic mixture</i> .....	29
3.2 METHODS .....	29
3.2.1 HERBS .....	29
3.2.1.1 <i>Preparation of water extract of herbs</i> .....	29
3.2.2 YOGURT .....	30
3.2.2.1 <i>Preparation of starter culture</i> .....	30
3.2.2.2 <i>Herbal yogurt preparation</i> .....	30
3.2.2.3 <i>Time Interval specifications</i> .....	30
3.2.3 MEASUREMENT OF pH AND TITRATABLE ACIDITY (TA) .....	31
3.2.4 PREPARATION OF YOGURT WATER EXTRACT.....	31
3.2.5 MEASUREMENT OF 1, 1-DIPHENYL-2-PYCRYLHYDRAZYL (DPPH).....	32
3.2.6 ORGANOLEPTIC ASSESSMENT .....	32
3.2.7 ENUMERATION OF VIABLE CELL (CFU) IN YOGURT .....	32
3.2.7.1 <i>Lactobacillus ssp</i> .....	32
3.2.7.2 <i>Streptococcus thermophilus</i> .....	33
3.2.7.3 <i>Observing yogurt viable cells by Scanning Electron Microscopy (SEM)</i> ...	33
3.2.8 <i>HELICOBACTER PYLORI</i> BACTERIA .....	34
3.2.8.1 <i>Bacterial isolation</i> .....	34
3.2.8.2 <i>Preservation of strains</i> .....	35
3.2.9 MEASUREMENT OF <i>H. PYLORI</i> GROWTH: DISK DIFFUSION METHOD (DDM).....	35
3.2.10 MEASUREMENT OF <i>H. PYLORI</i> GROWTH: MINIMUM INHIBITORY CONCENTRATION (MIC) TEST .....	35
3.2.10.1 <i>Inoculum preparation</i> .....	36
3.2.10.2 <i>Inoculation of agar plates</i> .....	36
3.3 STATISTICAL ANALYSIS .....	37
CHAPTER 4: RESULTS .....	38
4.1 EFFECT OF HERBAL YOGURT ON THE CHANGES OF pH.....	38
4.2 EFFECT OF HERBAL YOGURT ON THE CHANGES OF TA .....	39

4.3 ANTIOXIDANT ACTIVITY IN HERBAL YOGURTS.....	40
4.4 VIABLE CELL COUNTS IN HERBAL-YOGURT.....	40
4.5 SENSORY EVALUATION OF HERBAL YOGURTS .....	43
4.6 <i>HELICOBACTER PYLORI</i> .....	43
4.6.1 <i>Morphology and identification of H. pylori</i> .....	44
4.7 EFFECTS OF EXTRACTS OF HERBS AND HERBAL-YOGURTS ON <i>H. PYLORI</i> GROWTH ..	46
CHAPTER 5: DISCUSSION.....	49
5.1 CHANGES IN THE FERMENTATION CHARACTERISTICS OF MILK BY HERBS.....	49
5.2 EATING AND NUTRITIONAL VALUE OF HERBAL-YOGURTS .....	50
5.2.1 <i>Sensory assessment</i> .....	50
5.2.2 <i>Antioxidant activities of herbal-yogurts</i> .....	52
5.2.3 <i>Yogurt fermentation and the predigestion of milk</i> .....	53
5.3 FUNCTIONAL VALUE OF HERBAL-YOGURTS .....	55
5.3.1 <i>“Gut-friendly” yogurt bacteria</i> .....	55
5.3.2 <i>Anti H. pylori activity</i> .....	57
5.4 GENERAL DISCUSSION.....	60
5.5 CONCLUSION .....	61
REFERENCES.....	62
APPENDICES .....	89
APPENDIX 1: EXPERIMENTAL DATA.....	89
1.1 <i>pH Analysis</i> .....	89
1.2 <i>Titrateable Acidity (TA) Analysis</i> .....	93
1.3 <i>Viable Cell Count Analysis (CFU)</i> .....	97
1.4 <i>DPPH Assay</i> .....	101
1.5 <i>Bacterial Growth Inhibition Assay</i> .....	104
APPENDIX 2: MEDIA PREPARATION AND REAGENTS .....	108
2.1 <i>Buffered Peptone Water</i> .....	108
2.2 <i>M17 Agar</i> .....	108
2.3 <i>MRS Agar</i> .....	108
2.4 <i>Mueller Hinton (MH) Blood Agar</i> .....	109
2.5 <i>Brain Heart Infusion Broth (BHIB)</i> .....	109
2.6 <i>NaOH Solution (1%)</i> .....	109
2.7 <i>Phenolphetalein (1%)</i> .....	109
2.8 <i>1, 1-diphenyl-2-pycrylhydrazyl (DPPH) Stock Solution</i> .....	109
2.9 <i>Gram-staining</i> .....	110
LIST OF PUBLICATIONS .....	111



# List of Figures

Figure	Page
FIGURE 2.1 <i>CINNAMON ZEYLANICUM</i> BARK .....	16
FIGURE 2.2 LICORICE PLANT, ROOT AND FRUIT.....	18
FIGURE 2.3 <i>ALLIUM SATIVUM</i> (GARLIC) .....	20
FIGURE 2.4 PEPTIC AND DUODENAL ULCERS IN HUMAN. ....	24
FIGURE 2.5 GASTRIC ULCER DEVELOPMENTS: .....	26
FIGURE 4. 1 EFFECTS OF HERBS ON THE CHANGES OF pH IN YOGURT DURING FERMENTATION AT 41°C .....	38
FIGURE 4. 2 EFFECTS OF HERBS ON THE CHANGES OF pH IN YOGURT DURING 28 DAYS OF STORAGE AT 4°C .....	38
FIGURE 4. 3 EFFECTS OF HERBS ON THE CHANGES OF TA IN YOGURT DURING THE FIRST 5 HOURS OF FERMENTATION AT 41°C.....	39
FIGURE 4. 4 EFFECTS OF HERBS ON THE CHANGES OF TA IN YOGURT DURING 28 DAYS OF STORAGE AT 4°C .....	39
FIGURE 4. 5 DPPH RADICAL INHIBITION CAPACITY OF WATER EXTRACTS FROM PLAIN AND HERBAL-YOGURTS DURING REFRIGERATED STORAGE .....	40
FIGURE 4.6 VIABLE CELL COUNT (CFU) OF <i>LACTOBACILLUS</i> SSP. IN PLAIN- AND HERBAL- YOGURTS DURING REFRIGERATED STORAGE (4°C).....	41
FIGURE 4.7 VIABLE CELL COUNT (CFU) OF <i>S. THERMOPHILUS</i> IN PLAIN- AND HERBAL- YOGURTS DURING REFRIGERATED STORAGE (4°C).....	42
FIGURE 4.8 LACTIC ACID BACTERIA (LAB) AS OBSERVED UNDER ELECTRON MICROSCOPE (MAGNIFICATION FROM LEFT TO RIGHT: X4900 AND X11800).....	42
FIGURE 4.9 <i>H. PYLORI</i> TRANSLUCENT COLONIES .....	44
FIGURE 4.10 GRAM-STAINING FROM PURE <i>H. PYLORI</i> CULTURE.....	44
FIGURE 4.11 RAPID UREASE TEST .....	45
FIGURE 4.12 CATALASE TEST.....	45
FIGURE 4.13 OXIDASE TEST .....	45

# List of Tables

Table	Page
TABLE 2.1 CLAIMED THERAPEUTIC AND BENEFICIAL EFFECTS OF PROBIOTIC BACTERIA IN HUMAN (FULLER, 1989; SHAH, 2007) .....	8
TABLE 2. 2 DESIRABLE PROPERTIES OF PROBIOTIC LACTIC ACID BACTERIA (ADOPTED FROM SALEMIN <i>ET AL.</i> , 1996; TANNOCK, 1997) .....	12
TABLE 2. 3 BIOCHEMICAL CHARACTERISTICS OF GENUS <i>H. PYLORI</i> (ADOPTED FROM FOX AND LEE, 1997) .....	25
TABLE 2. 4 VIRULENCE FACTORS IDENTIFIED IN <i>H. PYLORI</i> (ADAPTED FROM <i>ET AL.</i> , 1987 & 1988; HIGASHI <i>ET A.L</i> , 2002) .....	28
TABLE 4.1 SENSORY DESCRIPTORS AND SCORES* OF YOGURTS.....	43
TABLE 4.2 STRAINS USED IN THE STUDY, SOURCE AND PATIENTS DATA .....	43
TABLE 4.3 INHIBITION OF <i>H. PYLORI</i> STRAINS GROWTH BY VARIOUS PLANTS WATER EXTRACTS .....	46
TABLE 4.4 GROWTH INHIBITION OF <i>H. PYLORI</i> BY WATER EXTRACTS FROM YOGURT DURING 14 DAYS REFRIGERATED STORAGE .....	47
TABLE 4. 5 MINIMUM INHIBITORY CONCENTRATION (MIC) OF HERBS AND HERBAL-YOGURT WATER EXTRACT ON THE GROWTH OF <i>H. PYLORI</i> ISOLATES <sup>1</sup> .....	48

# LIST OF ABBREVIATIONS

BF	Before Fermentation
μl	Microlitre
ml	Millilitre
μg	Microgram
mg	Milligram
nm	Nanometer
mm	Millimeter
rpm	Revolution Per Minute
LAB	Lactic Acid Bacteria
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
TA	Titrateable Acidity
MIC	Minimum Inhibitory Concentration
DDM	Disk Diffusion Method
cfu	Colony Forming Unit
dH <sub>2</sub> O	Distilled Water
BHIB	Brain Heart Infusion Broth
<i>S. thermophilus</i>	<i>Streptococcus thermophilus</i>
<i>L. acidophilus</i>	<i>Lactobacillus acidophilus</i>
<i>H. pylori</i>	<i>Helicobacter pylori</i>
GI	Gastrointestinal